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Tyrosine Phosphorylation of a c-Src-Like Protein Is Increased in Membranes of CD4⁻ CD8⁻ T Lymphocytes from *lpr/lpr* Mice

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Mice homozygous for the autosomal recessive *lpr* gene have a disorder that results in autoimmunity and massive accumulation of T lymphocytes lacking CD4 and CD8 surface markers. These abnormal T cells exhibit constitutive tyrosine phosphorylation of a component of the CD3-T-cell receptor complex. We compared membrane tyrosine phosphorylation in *lpr/lpr* CD4⁻ CD8⁻ T cells and control T cells. *lpr* membranes exhibited a 7.3-fold increase ($n = 16$) in tyrosine phosphorylation of a 60-kilodalton protein. The increase was correlated with the Lpr but not the CD4⁻ CD8⁻ phenotype in that p60 phosphorylation was not increased in membranes from normal CD4⁻ CD8⁻ thymocytes. To identify the p60 in *lpr* cells, we examined the activity of several T-cell tyrosine-specific protein kinases. $p56^{lck}$ phosphorylation was only slightly increased in *lpr* membranes (2.2-fold; $n = 16$). Phorbol ester treatment of intact T cells before membrane isolation caused $p56^{lck}$ to migrate as $pp60^{lck}$; however, $pp60^{lck}$ could be clearly distinguished from the pp60 in *lpr* cells by two-dimensional gel electrophoresis. The pp60 from *lpr* cells exhibited several isoforms at pH ~6.3 to 6.5. Although on two-dimensional gels $pp60^{c\text{-}src}$ had a pI (6.4 to 6.8) within a similar region, $p60^{c\text{-}src}$ mRNA, protein, and kinase activities were not increased in *lpr* cells. In addition, staphylococcal V8 proteolytic cleavage of the *lpr* pp60 isolated on two-dimensional gels yielded two major fragments, a pattern distinct from that of $pp60^{c\text{-}src}$. However, by using an antiserum against the C-terminal sequence of c-Src and other related kinases, including $p59^{fyn}$, the pp60 could be immunoprecipitated in greater amounts from *lpr* than from control T cells. When $pp59^{fyn}$ was selectively immunoprecipitated from T-cell membranes with specific antisera, its molecular weight, proteolytic cleavage pattern, and behavior on two-dimensional gels were identical to those of the pp60 from *lpr* cells. We conclude that $p59^{fyn}$ phosphorylation is increased in membranes from *lpr/lpr* CD4⁻ CD8⁻ T cells and that the increase is correlated with constitutive tyrosine phosphorylation and perhaps with the expansion of this unusual T-cell population.

The massively enlarged lymphoid tissue in *lpr/lpr* mice is populated by abnormal lymphocytes expressing an array of markers associated with lymphocyte activation (3). Since these cells lack L3T4 and Lyt-2 (CD4⁻ CD8⁻) (6, 41, 58), it is the presence of Thy-1 and the productive rearrangement of the T-cell receptor genes (16, 43) that suggest a T-cell origin. These abnormal CD4⁻ CD8⁻ or double-negative T (DNT) cells accumulate in great numbers in the peripheral lymphoid organs, coincident with development of a lupus erythematosus-like syndrome (26). Although the identity of the recessive *lpr* gene that causes this disorder is unknown, it is expressed intrinsically in T lymphocytes because the *lpr* phenotype is expressed only in cells of *lpr* origin when mixtures of *lpr* and normal bone marrow are given to irradiated hosts (30). DNT cells from *lpr* mice are known to express c-*myb* constitutively at uncharacteristically high levels (20, 42, 60), yet these cells are relatively refractory to activation in vitro when stimulated via the T-cell receptor or through other surface receptors (17). Thus, although these cells are not neoplastic, they appear to constitute an immature T-cell subset that does not proliferate rapidly either in vivo or in response to T-cell mitogens in vitro (29). These cells do, however, arise in the thymus and persist to accumulate in peripheral locations.

In many cell types, growth is regulated by surface receptors that either exhibit intrinsic tyrosine-specific protein kinase activity (59) or may be coupled indirectly to the regulation of tyrosine phosphorylation (40, 48). Altered growth (25, 27) and differentiation (39) are sometimes caused by mutation or aberrant regulation of tyrosine kinases. There is some indication that this may be the case in *lpr* DNT cells, since constitutive tyrosine phosphorylation of p21, a component of the T-cell receptor-CD3 complex, has been detected (49). In normal T cells, this 21-kilodalton (kDa) substrate is phosphorylated on tyrosine residues only after T-cell activation by presented antigen or by antibodies to T-cell surface constituents (50). Although the tyrosine kinase that phosphorylates p21 has not been identified, the major tyrosine kinase in T cells, Lck, has been identified and sequenced (8, 9, 37, 56). In this report, we demonstrate that normal and *lpr* T cells exhibit at least three distinct tyrosine kinases in the 60-kDa region, $pp60^{c\text{-}src}$, $pp59^{fyn}$, and $pp60^{lck}$, a form of $pp56^{lck}$ that results from activation of protein kinase C. This last 60-kDa species has also been observed by others (10, 55). We also show that phosphorylation of $p59^{fyn}$ is increased by sevenfold in membranes from *lpr* DNT cells. The increase in $pp59^{fyn}$ is not characteristic of normal mature or immature thymic DNT cells but rather is correlated with the Lpr phenotype. Since the p21 component of CD3 is consistently phosphorylated in resting *lpr* DNT cells (49), our data also suggest that alterations in $p59^{fyn}$ activity may be related to p21 tyrosine phosphorylation.

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MATERIALS AND METHODS

Preparation of cells. Purified T lymphocytes were prepared from single-cell suspensions of various lymphoid tissues from MRL/Mp$^-$ *lpr/lpr* (MRL/*lpr*) and MRL/Mp+/+ (MRL/+) mice. The latter strain shares the MRL genetic background but lacks the *lpr* gene (3). Both strains are maintained at our colony at the University of North Carolina. Briefly, B cells were removed from the single-cell suspensions by panning on plates coated with goat anti-mouse immunoglobulin M. Cells were then treated with cytolytic immunoglobulin M antibody against CD4 (RL 172.4) (12) and CD8 (31M) (51), followed by incubation with added rabbit complement (Low-Tox M; Cedarlane, Westbury, N.Y.). Intact cells were separated from lysed cells by using lymphocyte separation medium (Organon Teknika, Durham, N.C.); greater than 90% viability and greater than 95% purity were achieved (29). Peripheral lymph nodes from *lpr* mice, the starting tissue for isolating *lpr* DNT cells, are initially ~80 to 90% DNT cells. Normal lymph node T cells were prepared similarly, but the lysis of CD4$^+$ and CD8$^+$ cells was omitted. Twenty adult thymuses were used to prepare DNT from MRL +/+ mice. Fetal thymocytes were harvested from fetuses from 16-day-pregnant mice. Approximately 10 thymuses were isolated per pregnant mouse. Fifty fetal thymuses were used to make a cell suspension of thymocytes. HSB cells were grown in RPMI 1640 with 10% fetal calf serum and harvested at a concentration of 10^6 cells per ml as described previously (18, 19).

Membrane preparations and phosphorylation. Cells were homogenized with a Polytron apparatus in 10 mM phosphate–1 mM EDTA (pH 7.4) with 20 μg of phenylmethylsulfonyl fluoride (PMSF), 10 μg of leupeptin, and 100 Kallikrein inhibitor units of aprotinin per ml. Subsequently, an equal volume of 0.5 M sucrose in phosphate-EDTA with 20 μg of PMSF and 10 μg of leupeptin per ml was added, and the nuclei were pelleted at 1,000 × *g* for 5 min. The supernatant was centrifuged at 105,000 × *g* for 60 min, and crude membrane fractions were resuspended in phosphate-buffered saline (pH 7.4) containing leupeptin (10 μg/ml) and PMSF (20 μg/ml). Membrane protein (50 μg) was phosphorylated in the presence of 1 mM $MnCl_2$, 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 7.0)–100 μM sodium vanadate–1 μM [γ-^{32}P]ATP (5 μCi per assay) in the presence or absence of 0.6% Triton X-100. T-cell membranes phosphorylated with Triton X-100 demonstrate autophosphorylation of p60$^{c\text{-}src}$, p60^{lck}, and p56^{lck}, a finding that has been confirmed by comparing *Staphylococcus aureus* V8 (Staph V8) cleavage maps of pp56 and pp60 from membrane autophosphorylation experiments using the cleavage maps of pp56^{lck} and pp60$^{c\text{-}src}$ immunoprecipitated with specific antisera (unpublished results). Phosphorylated proteins were subjected to 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Gels were stained, destained, dried, and autoradiographed, using Kodak XAR or XRP film. When indicated, dried gels were incubated with 1 M KOH for 2 h at 55°C, placed back into destaining solution, redried, and reautoradiographed.

Phosphoamino acid analysis. To analyze the phosphorylated tyrosine content in p56 and p60, an autoradiograph was used to identify the p56 or p60 bands for excision. Phosphoprotein was electroeluted into a dialysis bag, using a tube gel apparatus. After trichloroacetic acid precipitation in the presence of carrier bovine serum albumin, phosphoprotein was hydrolyzed in 6 N HCl at 100°C for 3 h in an N_2 atmosphere. Hydrolyzed amino acids were resuspended, and phosphoamino acid standards (Sigma Chemical Co.) were added. The separation was accomplished by two-dimensional (2-D) phosphoamino acid analysis on thin-layer chromatography plates as described previously (19).

Immunoprecipitation. Membrane protein (300 to 500 μg) was immunoprecipitated with monoclonal antibodies or antisera either before or after phosphorylation. When indicated, membrane fractions were phosphorylated in the presence of 1 mM Mn^{2+}–20 mM PIPES (pH 7.0)–200 μM vanadate–0.25 μM [γ-^{32}P]ATP (60 μCi per assay) in a total volume of 400 μl. The assay was terminated with 2× RIPA buffer (400 μl), yielding final concentrations of 5 mM EDTA, 500 μM vanadate, and 20 mM *p*-nitrophenyl phosphate. After lysis, the supernatants were clarified, transferred, and incubated for 45 min at 0°C with 2 μl of anti-c-Src monoclonal antibody 327 (35) (generously provided by Joan Brugge), anti-c-Src monoclonal antibody GD11 (45) (generously provided by Sarah Parsons), α-c-Src, a rabbit polyclonal antibody against a c-Src-like C-terminal peptide, STEPQYQP GENL (generously provided by D. Michael Payne and Michael Weber), or a specific p59$^{c\text{-}fyn}$ antiserum raised against a TrpE fusion protein containing residues 26 to 74 of murine p59^{fyn} (generously provided by Roger Perlmutter). Pansorbin, either alone (for α-c-Src and anti-p59^{fyn} immunoprecipitations) or preincubated with rabbit anti-mouse immunoglobulin, was added to the mixture and incubated for at least 30 min. Complexes were pelleted and washed sequentially in RIPA buffer containing 1 M NaCl, 0.15 M NaCl, and no NaCl. After the last wash, immunoprecipitates were boiled in SDS sample buffer and analyzed by SDS-PAGE. When immunoprecipitates were to be analyzed by 2-D gel electrophoresis, 30 μl of H_2O was added to the pellet, and the mixture was boiled for 5 min. After cooling, 30 μl of 2× lysis buffer containing 9 M urea and Sigma ampholines (80%, pH 5 to 7; 20%, pH 3 to 10) was added, the Pansorbin was pelleted, and the supernatant containing phosphoproteins was transferred to a new tube.

Isolated human platelets were lysed in RIPA buffer and immunoprecipitated with monoclonal antibody GD11 or 327 as detailed above. The final pellet was washed twice with buffer containing 1 or 10 mM Mn^{2+}, 50 mM PIPES (pH 7.0), and 200 μM vanadate. The assay mix (30 μl) was brought to 1 μM ATP (10 μCi of [γ-^{32}P]ATP), and the immunoglobulin complex was phosphorylated for 5 min at 21°C.

Two-dimensional gel electrophoresis and Cleveland mapping. Two-dimensional gels were performed essentially as described by O'Farrell (44). Samples of 25 μl were loaded onto tube gels in the 2-D lysis buffer described above. The tube gels had been preequilibrated by electrophoresis with ampholines. Equilibrium isoelectric focusing was then performed for 18 h at 400 V. Tube gels were then extruded into a 15-ml tube and equilibrated for 2 h in sample buffer, after which they were laid on an 8% SDS-polyacrylamide gel and electrophoresed for 18 h. The pH gradient in 2-D gels was determined by isolating gel slices throughout the gradient and determining the pH after elution in water. Dried gels were subjected to KOH treatment when indicated.

Staph V8 cleavage mapping was performed as previously described (19, 24). Areas of interest from one-dimensional (1-D) or 2-D gels were identified by overlaying autoradiograms and gels. Excised gel pieces were stuffed in the well of a 5% stacking gel with 50 or 200 ng of Staph V8 protease. Protease and phosphoprotein were run into the stacking gel, and the current was turned off for 30 min. Current was reapplied, and the electrophoresis was carried out into either

a 15% SDS-gel or a 5 to 15% gradient gel for 5 h at 200 V at 4°C.

RNA analysis. T-cell subpopulations and lines were prepared and lysed in guanidinium isothiocyanate, and poly(A)-selected RNA was isolated as described previously (4). After formamide-agarose gel electrophoresis, the RNA was transferred to nitrocellulose and hybridized. A *Bam*HI-*Pst*I fragment from plasmid p330 which contained the mouse c-*src* cDNA (generously provided by Ricardo Martinez, Massachusetts Institute of Technology) was nick translated and used for hybridization (38). The quantity of RNA loaded per gel lane was assessed after blots were stripped and reprobed with γ-actin cDNA.

Western blotting. c-Src protein was immunoprecipitated with monoclonal antibody GD11 from 500 μg of membrane protein isolated from *lpr* DNT cells or MRL +/+ lymph node T cells. Immunoprecipitation was performed as described above, and the immune complex was boiled in 30 μl of H_2O and prepared for 2-D gel electrophoresis as described above. The 8% PAGE (second dimension) was performed by using prestained molecular weight markers (Bio-Rad Laboratories). The p50-to-p75 region of the 2-D gel was cut out as a strip and transferred to nitrocellulose, using a Bio-Rad transblot apparatus. Filters were preincubated overnight in 1% Carnation milk–10 mM Tris hydrochloride (pH 7.5)–170 mM NaCl. The antibody-binding buffer contained 0.25% gelatin, 0.5% milk, 0.02% sodium azide, and monoclonal antibody 327 in 10 mM Tris hydrochloride (pH 7.5)–170 mM NaCl–0.05% Nonidet P-40. Washes were performed in 10 mM Tris hydrochloride (pH 7.5)–170 mM NaCl–0.5% gelatin–0.05% Nonidet P-40. The final wash buffer eliminated the gelatin. Rabbit anti-mouse immunoglobulin G (Jackson Immunoresearch Laboratories) and ^{125}I-labeled *S. aureus* protein A (Dupont, NEN Research Products) were used to develop the Western blot (immunoblot). After washing, autoradiography on Kodak XAR film was performed.

RESULTS

***lpr* T-cell membranes exhibit increased tyrosine phosphorylation of p60.** Tyrosine kinase activity was assessed in membrane fractions from lymph node DNT cells isolated from MRL/*lpr* mice and from various T-cell populations isolated from MRL/+ mice. The latter strain is congenic with MRL/*lpr* but expresses wild-type alleles at *lpr*. Figure 1 shows an autoradiograph from an alkali-treated SDS gel of membrane phosphoproteins. Addition of Triton X-100 to the membrane phosphorylation reaction stimulated the phosphorylation of three major alkali-resistant phosphoproteins, p56, p60, and p116. Phosphoamino acid analysis of p56 and p60 eluted from gels before alkali treatment confirmed that both exhibited >80% phosphorylated tyrosine in *lpr* membranes and >60% in controls. The most striking difference between *lpr* DNT and normal lymph node T cells was an increase in p60 phosphorylation (Fig. 1). Densitometry of autoradiographs revealed that maximal p60 phosphorylation in *lpr* membranes was 7.30 ± 1.79-fold (mean ± standard error of the mean; $n = 16$) greater than in MRL/+ splenic or lymph node T-cell membranes. Phosphorylated p56^{lck} in *lpr* membranes was either equivalent to or only slightly higher than that of normal T-cell membranes (a mean increase of 2.22 ± 0.55-fold; $n = 16$). The increase in phosphorylation of *lpr* p60 was significantly greater than that of p56^{lck} ($P < 0.002$; paired t test). The ratio of pp60 to pp56^{lck} in *lpr* DNT cell membranes ranged from 1:2 to 2:3. The ratio in membranes isolated from normal lymph node T cells was 1:4 to 1:7 and was consistent whether the control T cells were purified from peripheral nodes, thymus, or spleen.

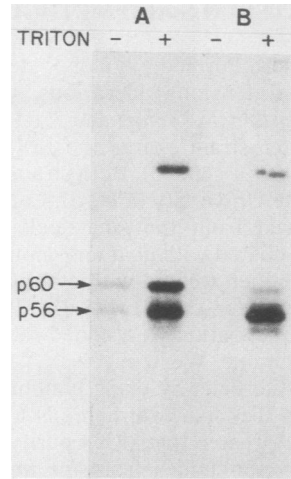

FIG. 1. Increased p60 phosphorylation in *lpr* DNT. Purified lymphocyte populations were prepared from single-cell suspensions of MRL/*lpr* (A) and control MRL/+ (B) lymph nodes as described in Materials and Methods. After complement lysis, *lpr* DNT cells were 99% CD4$^-$ CD8$^-$ and were >95% Thy-1$^+$. Cells were homogenized in 10 mM phosphate buffer–1 mM EDTA (pH 7.4) with a Polytron apparatus, and a particulate fraction was prepared. T-cell membranes were phosphorylated with 1 mM Mn^{2+}–50 mM PIPES (pH 7.0)–100 μM vanadate–1 μM [γ-^{32}P]ATP (5 μCi per assay) in either the absence (−) or presence (+) of 0.6% Triton X-100 (Triton X-100 treatment produces maximal tyrosine phosphorylation of lymphocyte membrane proteins). Phosphorylated proteins were subjected to 8% SDS-PAGE. The autoradiograph is of a dried gel that had been treated with 1 M KOH at 55°C for 2 h.

Enhanced p60 phosphorylation is observed in *lpr* DNT but not in normal DNT cells from thymuses. To determine whether enhanced p60 phosphorylation was found in normal DNT populations, DNT cells were isolated from the thymuses of 20 normal adult mice, in which normal adult DNT cells are known to represent <2% of thymocytes (21). Membranes were prepared and phosphorylated. Densitom-

TABLE 1. Quantitation of pp56 and pp60 phosphorylation in T-cell populations[a]

Protein	*lpr* DNT	LNT	Thymocytes	Adult DNT
pp56	15.35, 15.55	11.89, 11.05	1.14, 0.93	2.26, 2.08
pp60	8.39, 8.54	2.54, 2.30	0.22, 0.14	0.33, 0.29
Ratio, pp60/pp56	1:1.8, 1:1.8	1:4.7, 1:4.8	1:5.2, 1:6.6	1:6.8, 1:7.2

[a] Membranes from *lpr* DNT cells, +/+ lymph node T cells (LNT), thymocytes, and adult DNT cells were prepared and phosphorylated as described in the legend to Fig. 1 and Materials and Methods. After 8% SDS-PAGE, gels were dried, treated with KOH, and exposed to Kodak XAR film. After autoradiography, densitometric tracings of pp56^{lck} and pp60 were made with an LKB densitometer with peak integration. The peak areas from each lane of duplicate samples are shown. Both the absolute amount and the ratio of p60 phosphorylation were increased in MRL/*lpr* DNT-cell membranes. Results are for one of two similar experiments.

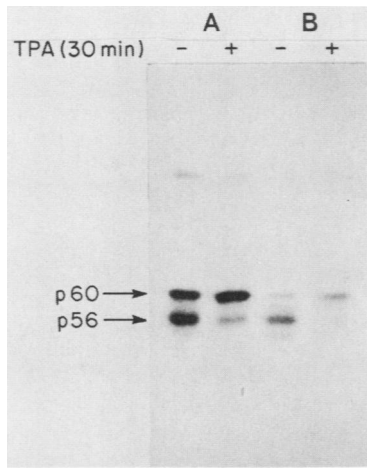

FIG. 2. Effect of TPA on membrane phosphorylation in mouse T cells. *lpr* DNT cells (A) and control lymph node T cells (B) were purified and incubated in culture for 30 min with (+) or without (−) 100 nM TPA. Cells were homogenized at 0°C, and membranes were isolated, phosphorylated, and analyzed by SDS-PAGE as described for Fig. 1. All samples shown were phosphorylated in the presence of 0.6% Triton X-100. The autoradiograph is of a dried gel that had been treated with 1 M KOH at 55°C for 2 h.

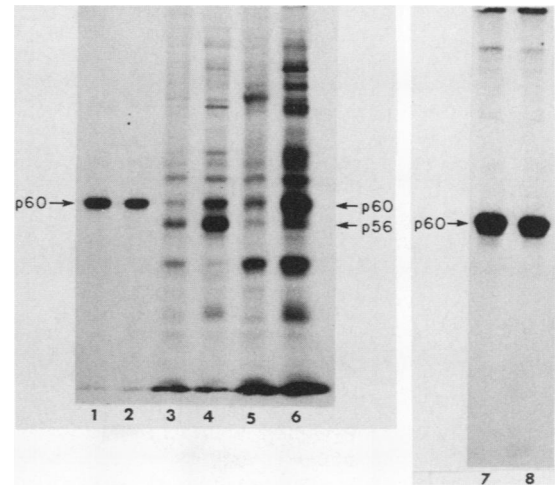

FIG. 3. Immunoprecipitation of pp60$^{c\text{-}src}$ from HSB cells incubated with (lanes 1, 5, 6, and 8) or without (lanes 2 to 4 and 7) 100 nM TPA before membrane preparation. Membranes (50 μg) were phosphorylated either with (lanes 4 and 6) or without (lanes 3 and 5) Triton X-100 and placed directly in SDS sample buffer; alternatively, membranes (400 μg) were phosphorylated with Triton X-100, followed by immunoprecipitation with either monoclonal antibody GD11 (lanes 1 and 2) or 327 (lanes 7 and 8) as described in Materials and Methods. Autoradiographs of 8% SDS-polyacrylamide gels are shown.

etry of pp56 and pp60 from gel autoradiographs showed a fourfold increase in pp60 in *lpr* T cells (Table 1). The pp60-to-pp56 ratio in *lpr* membranes was <1:2, but in normal T cells the ratio was 1:5. In the normal population of thymocytes as well as in the purified thymic DNT cells, the ratio was 1:6. The diminished amount of total p56 and p60 phosphorylation in the thymic populations was characteristic of all thymocytes and will be the subject of another report. A similar result (pp60-to-pp56 ratio of 1:6) was obtained with fetal thymocytes (85% CD4$^-$ CD8$^-$) (11) purified from 16-day-gestation fetal mice (data not shown). Thus, both normal fetal DNT cells, which are considered to be less mature, and adult DNT cells have the normal p60 phosphorylation pattern. Only *lpr* DNT cells showed increased p60 phosphorylation.

Identification of p60: TPA treatment results in a pp56^{lck}→pp60^{lck} shift. *lpr* DNT cells are relatively refractory to activation; i.e., their proliferative response to 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and the calcium ionophore A23187 is significantly less than that of normal T cells (29). To determine whether the reduced response was related to altered membrane phosphorylation, we incubated purified *lpr* DNT cells and normal T cells with TPA (100 nM) and A23187 (1 μM) for 30 min before isolating membranes. Whereas A23187 had no effect, TPA resulted in decreased phosphorylation of p56 and a slight increase in p60 phosphorylation. Both *lpr* and normal T cells had qualitatively similar responses (Fig. 2).

When the experiment described above was first performed, it was not known that TPA treatment resulted in a mobility shift of p56^{lck} to p60^{lck} (10, 55). Consequently, we tried to determine whether TPA was inhibiting p56^{lck} activity and activating another tyrosine kinase, p60$^{c\text{-}src}$. Membranes were isolated from TPA-treated and control HSB cells, a human T-cell line. After phosphorylation, pp60$^{c\text{-}src}$ was immunoprecipitated with one of two specific monoclonal antibodies, GD11 or 327. p60$^{c\text{-}src}$ phosphorylation was not increased by TPA (Fig. 3). In a further attempt to identify the p60 whose phosphorylation was enhanced by TPA pretreatment, we performed 2-D gel electrophoresis. The increase in pp60 appeared to result from a change in the electrophoretic characteristics of p56^{lck} (Fig. 4). Activation of protein kinase C initiated a process that retarded the electrophoretic mobility of p56^{lck} while slightly shifting the pI (from pH ~6.2 to ~6.0) as if phosphate groups had been added. We confirmed that the pp56 and pp60 isoforms at pH ~6.0 to 6.2 were Lck by performing 2-D gel electrophoresis and proteolytic cleavage maps after immunoprecipitation using specific p56^{lck} antisera (unpublished results). The other group of p60 phos-

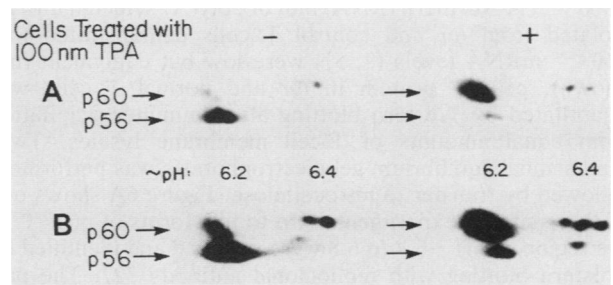

FIG. 4. Two-dimensional gel electrophoresis of phosphorylated membranes isolated from HSB cells incubated with or without 100 nM TPA. Intact cells were treated for 30 min and homogenized, and membranes were isolated and phosphorylated. The reaction was stopped in lysis buffer with urea and ampholines. Equilibrium isoelectric focusing was followed by 8% PAGE and autoradiography. The lighter exposure of the gels (A) shows pp56^{lck} at pH ~6.1 to 6.2; pp60^{lck} extended to pH 6.0. The pp60 isoforms that were not pp60^{lck} were found at pH ~6.3 to 6.5. A longer exposure of this autoradiograph (B) more clearly reveals an additional acidic isoform of the p60 phosphoproteins at pH ~6.3 to 6.5 in cells pretreated with TPA. The autoradiograph is of a dried gel that had been treated with 1 M KOH at 55°C for 2 h.

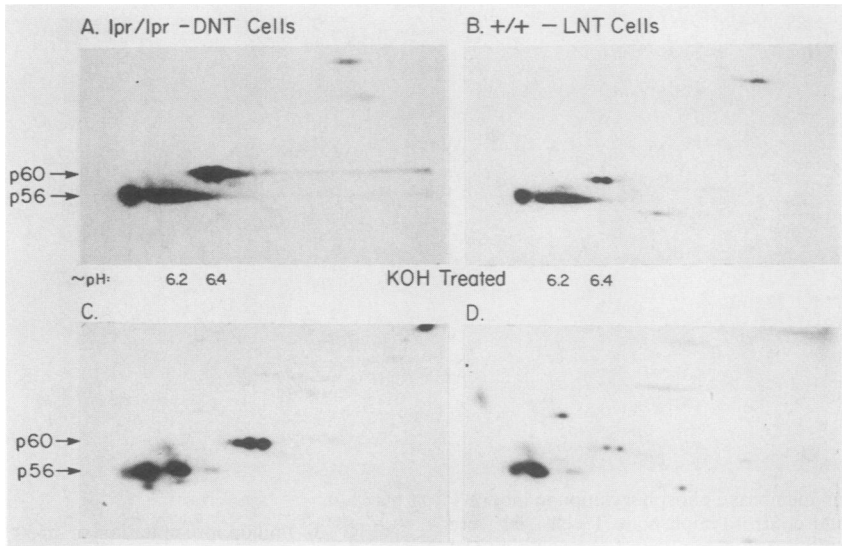

FIG. 5. Two-dimensional gel electrophoresis of membrane phosphoproteins demonstrating that the p60 whose phosphorylation was increased in *lpr* cells had an isoelectric point of pH ~6.3 to 6.5. MRL/*lpr* DNT cells (A and C) and MRL/+ lymph node T cells (B and D) were isolated. Membranes were prepared and phosphorylated. Equilibrium isoelectric focusing was followed by 8% PAGE. Gels were dried and either subjected to KOH treatment (A and B) or not (C and D), and autoradiography was performed.

phoproteins (at pH ~6.3 to 6.5) exhibited an additional acidic isoform when membranes from TPA-treated cells were phosphorylated (Fig. 4B). This effect of TPA may be analogous to the known TPA-dependent phosphorylation of $p60^{c\text{-}src}$ on serine 12 (23).

The pp60 in *lpr* cells was neither $pp60^{lck}$ nor $pp60^{c\text{-}src}$. Figure 5 shows the results of two separate experiments in which membranes from normal and *lpr* DNT cells were phosphorylated and subjected to 2-D gel electrophoresis. Increased p60 tyrosine phosphorylation was observed in the substrates that equilibrated at pH ~6.3 to 6.5 but not with $p60^{lck}$ at pH 6.0. We next immunoprecipitated $pp60^{c\text{-}src}$ from phosphorylated T-cell membranes with monoclonal antibody 327. When the immunoprecipitates were run on 2-D gels, they exhibited four to five isoforms with pIs of ~6.4 to 6.8 (data not shown).

We investigated whether the pp60 in *lpr* cells might be $pp60^{c\text{-}src}$. A Northern (RNA) blot of poly(A)-selected mRNA isolated from *lpr* and control T cells demonstrated that $p60^{c\text{-}src}$ mRNA levels (2, 57) were low but equivalent (not shown). $p60^{c\text{-}src}$ protein in *lpr* and normal T cells was quantitated by Western blotting after immunoprecipitation from equal amounts of T-cell membrane lysates. Two-dimensional equilibrium gel electrophoresis was performed, followed by transfer to nitrocellulose. Figure 6A shows one of three similar experiments. Up to five forms of $p60^{c\text{-}src}$ in the region of pH ~6.4 to 6.8 were resolved and identified by Western blotting with monoclonal antibody 327. The patterns and amounts of $p60^{c\text{-}src}$ were similar in both *lpr* and control cells.

Although $p60^{c\text{-}src}$ was not overexpressed in *lpr* cells, $p60^{c\text{-}src}$ phosphorylation may have been increased as a result of an increase in specific activity. We therefore further characterized lymphocyte $pp60^{c\text{-}src}$ and the *lpr* pp60 by Staph V8 proteolytic mapping. Immunoprecipitated $pp60^{c\text{-}src}$ from membranes isolated from cells incubated with or without TPA was mapped along with $pp60^{lck}$ and $pp56^{lck}$ (Fig. 6B). $pp60^{c\text{-}src}$ showed a single fragment (~29 kDa in our gels) similar to the one seen by others studying $pp60^{c\text{-}src}$ phosphorylated in cell-free preparations (22). $pp60^{lck}$ and $pp56^{lck}$ were similar to each other. However, the pattern of $pp60^{lck}$ isolated from 1-D gels was more complex than that of $pp56^{lck}$, indicating that the p60 region contained more than one tyrosine substrate. Figure 6C shows the p29 fragment in $pp60^{c\text{-}src}$ immunoprecipitates from HSB, normal mouse (+/+) T cells, and *lpr* DNT cells. Finally, $p60^{c\text{-}src}$ was immunoprecipitated from equal amounts of *lpr* and normal T-cell phosphorylated membranes or from unphosphorylated membranes. The latter immunoprecipitates were used for an immune complex autophosphorylation assay. There was no reproducible difference between *lpr* and normal T-cell $p60^{c\text{-}src}$ phosphorylation (data not shown). Thus, $p60^{c\text{-}src}$ gene expression and activity were similar in *lpr* and normal T cells.

The p60 in *lpr* cells is the c-Src-like protein $p59^{fyn}$. A contrasting result was seen when membranes were phosphorylated and immunoprecipitated with α-c-Src, a polyclonal antiserum to the C-terminal Src peptide. The immunoprecipitates were subjected to 2-D gel electrophoresis, and the *lpr* membranes clearly yielded increased pp60 with a pI of 6.3 to 6.5 (Fig. 7). Since several members of the Src family, including $p59^{fyn}$ (32, 53) and $p56^{lck}$ (37, 56), have C termini that would be recognized by this antiserum, we concluded that the pp60 in *lpr* cells is c-Src like.

We then analyzed the cleavage pattern of the pH 6.3-to-6.5 p60 phosphoproteins in *lpr* cells by performing 2-D gel electrophoresis, excising the pH 6.3-to-6.5 phosphoproteins, and performing proteolytic cleavage maps. The *lpr* pp60 isoforms contained two major cleavage products (Fig. 8A). Figure 8B compares the 2-D gel-isolated pH 6.3-to-6.5 isoforms from mouse and human T cells with GD11-immunoprecipitated $p60^{c\text{-}src}$ from human platelets. The *lpr* isoforms had two fragments; $pp60^{c\text{-}src}$ had only one. To determine whether the c-Src-like p60 with pH 6.3-to-6.5 isoforms was $pp59^{fyn}$, mouse T-cell membranes were phosphorylated and immunoprecipitated with specific anti-$p59^{fyn}$ antisera. The Staph V8 cleavage map of immunoprecipitated $pp59^{fyn}$ from mouse normal lymph node T cells (Fig. 8C) or from *lpr* DNT cells (not shown) was identical to that of the pI 6.3-to-6.5 $p60^{c\text{-}src}$-like protein. In addition, immunoprecipitated

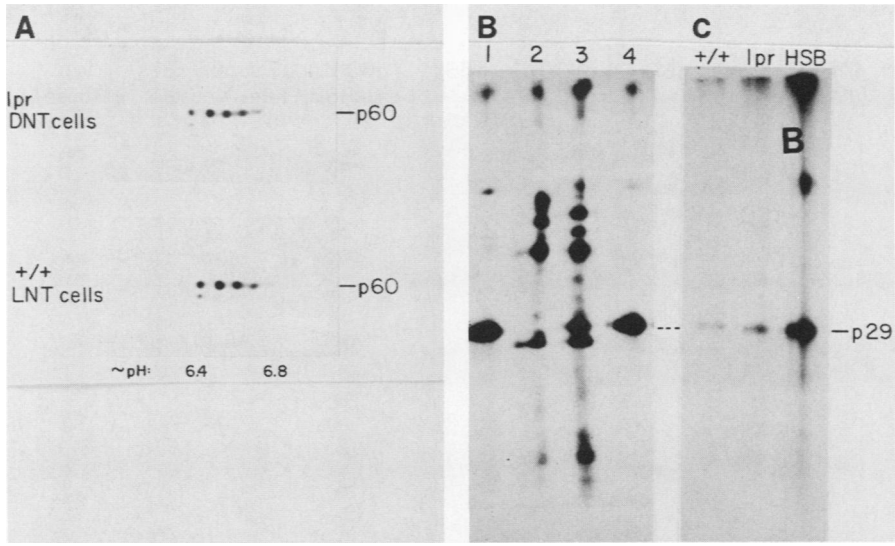

FIG. 6. Studies of $p60^{c\text{-}src}$ in T cells. (A) Equivalent amounts of immunoidentifiable c-Src are seen in *lpr* and normal T cells. $p60^{c\text{-}src}$ was immunoprecipitated with monoclonal antibody GD11 from 500 μg of MRL/*lpr* DNT-cell or control MRL/+ normal T-cell membranes after detergent lysis in RIPA buffer. Precipitates were prepared for 2-D gel electrophoresis. The p50-to-p75 region of the 2-D gel was cut out and transferred to nitrocellulose filters. The transfer was monitored with prestained molecular weight markers. Western blotting with monoclonal antibody 327 was performed, and ^{125}I-labeled *S. aureus* protein A was visualized by autoradiography on Kodak XAR film (2-day exposure, using a Cronex Lightning-Plus intensifier). Monoclonal antibody GD11 was used in excess, as demonstrated by the immunoprecipitation of >20-fold more $p60^{c\text{-}src}$ activity from human platelets under the same conditions (not shown). (B and C) Staph V8 proteolytic cleavage maps of samples from phosphorylated membranes and from monoclonal antibody 327 and GD11 immunoprecipitates of phosphorylated membranes. Samples phosphorylated in the presence of Triton X-100 were run on 8% SDS-polyacrylamide gels. Autoradiographs were used to cut out the indicated samples for cleavage mapping with 200 ng of Staph V8 protease. (B) GD11 immunoprecipitates ($pp60^{c\text{-}src}$) from membranes from TPA-treated (lane 1) or control (lane 4) cells yielded an equivalent single fragment. The pattern from $pp56^{lck}$ (lane 2) and the p60 region of a 1-D gel from membranes isolated from TPA-treated cells (lane 3) were similar, but the p60 map was more complex because it contained $pp60^{lck}$ as well as other p60 tyrosine kinases. (C) Monoclonal antibody 327 immunoprecipitates of phosphorylated membranes from +/+ normal mouse T cells, *lpr* DNT cells, and HSB human T cells. A single 29-kDa fragment was produced by 200 ng of Staph V8 protease.

$pp59^{fyn}$ from phosphorylated T-cell membranes had an isoelectric point at pH ~6.3 to 6.5 (Fig. 9A). The identity was confirmed by mixing immunoprecipitated $pp59^{fyn}$ with an equal portion of phosphorylated *lpr* DNT-cell membrane proteins and demonstrating that $pp59^{fyn}$ and the pp60 pH 6.3-to-6.5 phosphoprotein in *lpr* membranes had virtually identical patterns upon 2-D gel electrophoresis (Fig. 9B).

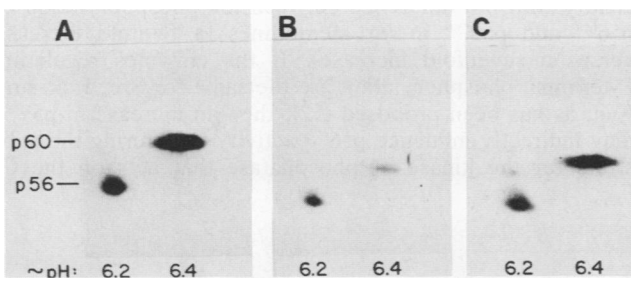

FIG. 7. Immunoprecipitation of phosphorylated membranes from *lpr* DNT cells (A), normal (+/+) mouse T cells (B), and HSB human T cells (C), using polyclonal α-c-Src antiserum, which recognizes the C-terminal peptide of c-Src-like proteins. Membranes (400 μg) were phosphorylated and immunoprecipitated as described in Materials and Methods. After final washes, the immunoprecipitates were prepared for 2-D gel electrophoresis, followed by equilibrium isoelectric focusing and 8% PAGE. Increased phosphorylation of pp60 at pH ~6.3 to 6.5 was seen in *lpr* cells and the tumor line, HSB. Immunoprecipitated $pp56^{lck}$ was seen in the pH 6.2 region.

DISCUSSION

These data indicate that membranes from *lpr* DNT cells exhibit a seven- to eightfold increase in p60 tyrosine phosphorylation. This phenomenon is characteristic of DNT cells from *lpr/lpr* mice but not of DNT cells from normal fetal or adult mice. We initially suspected that the increase in p60 was due to an increase in $p60^{lck}$, a form of $p56^{lck}$ that might be expected in a chronically but abortively activated immature T cell. This possibility was ruled out by the isoelectric focusing characteristics of $pp60^{lck}$. We next thought that the increased phosphorylation of p60 isoforms at pH ~6.3 to 6.5 represented increased c-Src activity, since two specific monoclonal antibodies (GD11 and 327) immunoprecipitated a tyrosine phosphoprotein from T cells that focused in this approximate region. The fact that $p60^{c\text{-}src}$ mRNA and protein were not elevated in *lpr* cells may simply have indicated that the specific activity of $p60^{c\text{-}src}$, not its absolute level, was elevated in *lpr* cells (see below). However, Staph V8 cleavage patterns distinguished the GD11- and 327-immunoprecipitated $pp60^{c\text{-}src}$ of T cells and platelets from the pp60 with a pI of ~6.3 to 6.5. The fact that the polyclonal antibody to the Src family C terminus recognizes the increased *lpr* p60 phosphoprotein emphasizes the relatedness of this protein to c-Src, while at the same time the cleavage map demonstrates its difference. The molecular weight, the recognition by the anti-$p60^{c\text{-}src}$ C-terminal antisera, and the indication by another group that Fyn is a 60-kDa tyrosine kinase in T-cell lines (54) suggested that the $p60^{c\text{-}src}$-like protein was $p59^{fyn}$. This $pp60^{c\text{-}src}$-related protein was recently cloned and se-

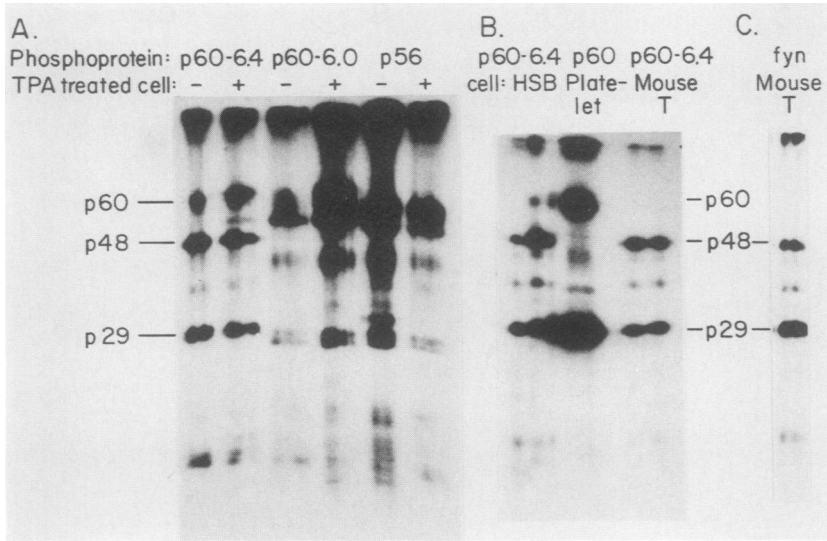

FIG. 8. Staph V8 proteolytic cleavage mapping of pp60^{lck}, pp60$^{c\text{-}src}$, and pp59^{fyn}. (A) Cleavage mapping was performed as described for Fig. 6 except that the samples had been subjected to 2-D gel separation before mapping. The pp60 from the region of pH 6.3 to 6.5 (designated p60-6.4) revealed two major fragments, p48 and p29, as well as undigested p60. pp56^{lck} and pp60^{lck} (designated p60-6.0) from the region of pH 6.0 to 6.2 are similar to each other. When cells had been treated with TPA, pp60^{lck} was increased and pp56^{lck} was decreased. (B) The pH 6.3-to-6.5 p60 phosphoproteins (designated p60-6.4) from 2-D gels of phosphorylated HSB and normal mouse T-cell membranes were mapped. In addition, pp60$^{c\text{-}src}$ was obtained by immunoprecipitating human platelets with monoclonal antibody GD11, followed by immune complex phosphorylation and 8% SDS-PAGE. Staph V8 cleavage (200 ng) of pp60$^{c\text{-}src}$ yielded one major (p29) fragment, while 2-D gel-separated pH 6.3-to-6.5 pp60 had two major fragments (p48 and p29). (C) p59^{fyn} was immunoprecipitated from phosphorylated membranes isolated from mouse T cells. Immunoprecipitates were run on 8% polyacrylamide gels, and pp59^{fyn} was excised and subjected to proteolytic cleavage mapping with 200 ng of Staph V8 protease.

quenced by two groups (32, 53). To confirm this supposition, we concentrated on the two unique features of the p60 whose phosphorylation was increased in *lpr* cells: (i) the pI of pH ~6.3 to 6.5 and (ii) the distinct Staph V8 cleavage map. Using specific antisera followed by 2-D gel and proteolytic cleavage, we showed that T-cell p59^{fyn} was identical to the p60$^{c\text{-}src}$-like protein in *lpr* cells. In addition, recent experiments have demonstrated an eightfold increase in Fyn protein on Western blots of *lpr* and normal mouse T cells (T. Katagiri, K. Urakawa, Y. Yamanashi, K. Semba, T. Takahashi, K. Toyoshima, T. Yamamoto, and K. Kano, submitted for publication).

The apparent increase in the p59^{fyn} protein in *lpr* cells may be related, directly or indirectly, to the constitutive tyrosine phosphorylation of the T-cell receptor-CD3 complex protein reported by others (49). Regulation of the activity of c-Src-like proteins is complex and is in part due to tyrosine phosphorylation of the c-Src-like molecules themselves (25, 27). For example, in intact normal cells, p60$^{c\text{-}src}$ is phosphorylated in the C-terminal region on Tyr-527 (14), a phosphorylation that inhibits the activity of the enzyme by up to 10-fold (14, 15). The constraint of p60$^{c\text{-}src}$ activity by Tyr-527 phosphorylation has been elegantly demonstrated by site-directed mutagenesis of Tyr-527, a substitution that yields mutants with enhanced tyrosine kinase activity and the ability to transform cells (7, 33, 46). It seems likely, albeit not proven, that the enzyme(s) that phosphorylates Tyr-527 is distinct from p60$^{c\text{-}src}$ itself (28).

It is possible that each of the Src family of tyrosine kinases is regulated in part by mechanisms similar to the inhibitory C-terminal phosphorylation of p60$^{c\text{-}src}$. Sites analogous to Tyr-527 phosphorylation are found in most members of this family, including Fyn (32, 53) and Lck (1, 36). We have clearly shown a difference between the phosphorylation of p56^{lck} and p59^{fyn} in *lpr* membranes (a twofold increase versus a sevenfold increase). If the enzymes regulating C-terminal phosphorylation are the same for Src, Lck, and Fyn, as has been proposed (52), then an increase in p59^{fyn} may indirectly influence p56^{lck} activity by altering the substrate for the kinase or phosphatase that acts on the C-

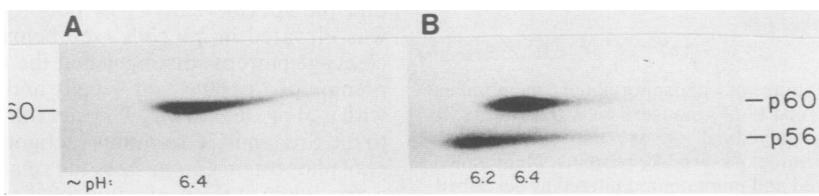

FIG. 9. Two-dimensional gel electrophoresis of T-cell membranes and immunoprecipitated pp59^{fyn}. *lpr* DNT-cell membranes were prepared and phosphorylated as described in Materials and Methods. (A) Phosphorylated membranes were immunoprecipitated with specific p59^{fyn} antisera and prepared for 2-D gel electrophoresis. pp59^{fyn} exhibited a pI at pH ~6.3 to 6.5. (B) The pp59^{fyn} immunoprecipitate electrophoresed alone in panel A was mixed with an equal volume of sample from phosphorylated *lpr* membranes that had been prepared for 2-D gel electrophoresis. pp56 was contributed solely by the membranes. The mixture of pp59^{fyn} and the pp60 at pH ~6.3 to 6.5 from *lpr* cells showed that they were indistinguishable by electrophoretic criteria.

terminal tyrosine. This could result in the modest elevation of $p56^{lck}$ activity observed in *lpr* cells. Conversely, alteration in c-Src-like protein activity can be regulated by means other than C-terminal tyrosine phosphorylation, as has been noted in tumor samples from colon (5), in other c-*src* mutants (31, 34), and at restricted times in the cell cycle (mitosis) (13).

It is tempting to speculate that overexpression of a $p59^{fyn}$ in *lpr* DNT cells results in the constitutive tyrosine phosphorylation of p21 in the T-cell receptor signaling complex (49). Moreover, this correlation may indicate that $p59^{fyn}$ is the kinase that phosphorylates p21 during normal T-cell receptor signaling. However, for the reasons noted above, an overexpressed kinase may only indirectly influence the phosphorylation of a specific substrate. The biologic defect in *lpr* cells appears to be an accumulation of a T-cell subset. This results in massive lymphadenopathy, yet the rate of growth of *lpr* DNT cells in the peripheral node is very low (47) and the isolated *lpr* DNT cells are hyporesponsive to normal T-cell mitogenic stimuli. It is plausible that a persistent intracellular signal, perhaps mediated by altered activity of a tyrosine kinase and characterized by consistent phosphorylation of p21, leads to a block in normal development pathways and an egress of these cells from the thymus. Further studies of the signaling pathway will be necessary to decipher whether $p59^{fyn}$ overexpression is central to the *lpr* phenotype.

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